Neuropathic pain often occurs after the injury of neurons in pain pathways, including that caused by diabetes and spinal cord injury. As only a limited number of medicines could suppress such chronic pain, appropriate or satisfactory pain therapy remains to be done in clinics (1).

Botulinum toxin type A (BoNT/A) is a botulinum neurotoxic protein produced by the bacterium *Clostridium botulinum*. BoNT/A is one of the most poisonous neurotoxins, but it is usually medically used for treatments of neuromuscular disorders because it is able to degrade the synaptosome-associated protein of relative molecular mass 25 K (SNAP-25), a protein required for synaptic vesicle fusion, and block the release of acetylcholine (ACh), which is the principal neurotransmitter at the neuromuscular junction, to cause the muscle-relaxing effects (2). Moreover, increasing clinical reports had demonstrated that BoNT/A also exhibits marked analgesic activity for chronic pain symptoms, including neuropathic pain (3).

BoNT/A is an effective denervation medicine, but in several clinical cases, the usage of BoNT/A is limited because of its possible motor dysfunctions (4). BoNT/A, as a protein complex, contains a neurotoxin (NTX) and non-toxic components. Recently, a NTX from *Clostridium botulinum* subtype A2 strain (named A2 NTX) was purified (5, 6), which was demonstrated to be a more potent neuromuscular blocker than the commercial BoNT/A product because of the absence of non-toxic components in A2 NTX (6). Considering that BoNT/A is a potent pain killer, in this study, we attempted to examine the possible pharmacological effects of A2 NTX for neuropathic pain.

Male ddY-strain mice (20 – 24 g; TEXAM, Nagasaki) were used. They were kept in a room maintained at 21°C ± 2°C and 55% ± 5% relative humidity with a 12-h light/dark cycle. Food and water were freely available. The procedures were approved by the Nagasaki University Animal Care Committee.

A2 NTX was generously provided by Chemo-Sero Therapeutic Research Institute (Kumamoto). It was dissolved in physiological saline [for intraplantar (i.pl.) treatment] or artificial cerebrospinal fluid (for direct application) just before administration.

The streptozotocin (STZ)-induced diabetic neuropathic pain model was generated as described previously (7). Mice were injected intravenously (i.v.) with STZ (200 mg/kg; Wako Pure Chemicals, Richmond, VA, USA).
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After 7 days, the plasma glucose level was measured using a glucose test kit (Ascensia®; Bayer, Tokyo). Only mice with plasma glucose concentration greater than 300 mg/dl were considered as diabetic mice.

Nociceptive tests, including mechanical paw pressure test, thermal paw withdrawal test, and electrical stimuli-induced paw withdrawal (EPW) test were performed according to our previous methods (8, 9).

The rotarod task performed in this study was modified from a previous report (10). Briefly, the mouse was put on the rotarod treadmill device (Muromachi Kikai Co., Ltd., Tokyo), and performance was assessed by measuring the latency until the mouse fell off the device. A cut-off time of 60 s was set in order to prevent motor damage.

Spinal cord injury was performed as previously described (11). The anesthetized mouse was immobilized to expose the dorsal surface of the dura mater, and spinal cord injury was made with 40 kdyn using a commercial device (Infinite Horizon Impactor; Precision Systems & Instrumentation, Lexington, KY, USA).

Results were analyzed with the least significant difference (LSD) test of an analysis of variance (ANOVA) and Student’s t-test. \( P < 0.05 \) was considered significant. All results are expressed as the mean ± S.E.M.

In BoNT/A-treated clinical cases, muscle weakness and paralysis are the most common adverse effects. In this study, to determine whether A2 NTX induces motor dysfunction, firstly, we performed a rotarod test, which is an established measurement for assessing sensitivity of drug-induced motor incoordination (10). A2 NTX (10 and 30 U/kg) was i.pl. injected into the naive mice. After 1 day, A2 NTX at both doses showed no change in the latency to fall at 6, 12, and 18 rpm. Similar results were also observed at 6 weeks (Fig. 1A). These results suggested that i.pl. injection of A2 NTX did not induce any motor dysfunction, indicating that A2 NTX may be a safe BoNT/A product. This finding was consistent with those of a recent report (6).

One of the pain models used in this study is the diabetes-induced neuropathic pain model, which was generated by i.v. injection of STZ at 200 mg/kg. Mice showed a remarkable increase of blood glucose levels (434.9 ± 20.8 mg/dl, \( n = 19 \)) 1 week after STZ-treatment, compared with vehicle-treated mice (136 ± 6.6 mg/dl, \( n = 18 \)). A2 NTX at different doses (1, 10, and 30 U/kg) and vehicle were i.pl. injected into the diabetic mice 1 week post STZ-treatment. At 1 week after these injec-

![Fig. 1.](image-url)

Fig. 1. Anti-allodynia effects of A2 NTX in peripheral diabetic neuropathic pain model. A: Neurological motor function in naive mice after A2 NTX–treatment (10 and 30 U/kg, i.pl.) through the rotarod tests. 10 and 30 indicate the doses of A2 NTX. B and C: STZ (200 mg/kg) was i.v. injected to induce diabetes. At 7 days after STZ-treatment, A2 NTX (1, 10, and 30 U/kg) were i.pl. injected. Paw pressure tests were performed at 1 week after A2 NTX–treatment (panel B) and at time-course points (panel C). The capital letter “V” indicates vehicle. PWT, paw withdrawal threshold. All data represent means ± S.E.M. from three or four mice. *\( P < 0.05 \), vs. the control-vehicle group; **\( P < 0.05 \), vs. the diabetes-vehicle group.
tions, vehicle-treated diabetic mice displayed significant reduction of pain thresholds in the mechanical paw pressure test, which was considered as mechanical allodynia. A2 NTX at 1, 10 and 30 U/kg significantly reversed this diabetes-induced mechanical allodynia (Fig. 1B). As 10 U/kg of A2 NTX showed maximal anti-allodynia actions, we adopted this dose in the following nociception tests. The diabetes-induced mechanical allodynia started at 1 week and lasted for at least 6 weeks after STZ-injection (Fig. 1C), being consistent with our previous results (7). The A2 NTX (10 U/kg)-treatment, which was given after the pain behavioral test on 1 week post STZ-injection, significantly reversed the allodynia from 2 weeks after STZ-injection (Fig. 1C). Considering the long-lasting anti-nociceptive effects and safety in motor function, A2 NTX would be a new candidate for treating diabetic neuropathic pain.

In the previous study, we developed an EPW test to distinguish Aβ-, Aδ-, and C-fibers by using different transcutaneous nerve stimuli at 2000-, 250-, and 5-Hz, respectively (8, 9). In this study, we also investigated the roles of different sensory fibers in diabetic neuropathic pain through this method. STZ caused significant decreases in the withdrawal thresholds in response to both 2000 Hz (Aβ-fiber) and 250 Hz (Aδ-fiber) stimuli 1 week after treatment, which lasted for at least 5 weeks. However, STZ did not change the withdrawal thresholds to 5 Hz (C-fiber) stimuli (Fig. 2: A – C). These results were consistent with the previous report by the Khan group (12) that Aβ- and Aδ-fibers, but not C-fiber, in diabetic rats developed abnormal spontaneous discharges and increased sensitivity to mechanical stimuli. They also reported that diabetic allodynia could not be prevented by the pretreatment with resiniferatoxin, a drug to deplete C-fiber afferents (12).

Although the mechanism of the anti-nociceptive action of A2 NTX is not yet clarified, Cui et al. (13) proposed the hypothesis that the inhibition of pain neurotransmitter release may be involved in the BoNT/A-induced suppression of the formalin-induced second phase of nociceptive responses in rats. As there is no significant change in the basal nociceptive threshold in BoNT/A-treated rats (13), however, this hypothesis needs to be further investigated. The lack of basal nociceptive threshold was also observed in the present study, in which A2 NTX–injected control mice showed no change in the pain thresholds.

Fig. 2. Reversal of A-fiber hypersensitivity in peripheral diabetic neuropathic pain by A2 NTX. A – C: Diabetes model was established by STZ (200 mg/kg, i.v.)-treatment. During pre-treatment to 5 weeks post-treatment, electrical stimuli–induced paw withdrawal test response to 2000- (panel A), 250- (panel B), and 5-Hz (panel C) stimulation was determined weekly. D – F: A2 NTX (10 U/kg) was i.pl. injected 1 week after STZ-treatment. At 1 week post A2 NTX–injection, electrical stimuli–induced paw withdrawal test response to 2000- (panel D), 250- (panel E), and 5-Hz (panel F) stimulation was determined. All data represent means ± S.E.M. from three to nine mice. *p < 0.05, vs. the control-vehicle group; †p < 0.05, vs. the diabetes-vehicle group.
responding to mechanical and electrical stimulations (Fig. 1C and Fig. 2: D – F).

On the other hand, A2 NTX (10 U/kg, i.pl.) administered 1 week post STZ-treatment significantly reversed this diabetes-induced A-fiber hypersensitization from 2 weeks, but never changed the thresholds to C-fiber stimuli (Fig. 2: D – F). Our previous study has reported that the transient receptor potential vanilloid receptor 1 (TRPV1), with predominant presence on C-fiber, was highly expressed on myelinated A-fibers in diabetic mice (7). In addition, there are papers that A2 NTX inhibited the membrane transport of TRPV1 through a degradation of soluble NSF-attachment protein receptor (SNARE, NSF: N-ethylmaleimide-sensitive fusion protein) proteins (14). According to these findings, it may be speculated that the peripheral treatment with A2 NTX reverses the A-fiber-selective abnormal pain in the diabetic pain model through a blockade of membrane transport of newly expressing and pain-related membrane proteins. Thus, we have proposed that A2 NTX may have two distinct antinociceptive mechanisms, one involves the inhibition of neurotransmitter release, and the other is the regulation of pain-related membrane proteins.

In this study, we also evaluated the role of A2 NTX in central neuropathic pain using the spinal cord injury model. In this model, 1 U/kg of A2 NTX was immediately applied into the exposed injured spinal cord. On the next day after injury, all of the injured mice, including A2 NTX–treated mice, exhibited very short latency to fall in rotarod tests, which were gradually recovered in an A2 NTX–independent manner until 10 and 14 days post-injury in rotarod tests at 6 and 12 rpm, respectively (Fig. 3: A and B). Although A2 NTX slightly, but significantly deteriorated the motor dysfunction at day 5 as well as days 5 and 10 in rotarod experiments using 6 and 12 rpm, respectively (Fig. 3: A and B), in both experiments, A2 NTX had no significant effects on motor dysfunction at day 14, when nociception tests started. After motor function recovery, spinal cord–injured mice showed serious long-term thermal hyperalgesia and mechanical allodynia, but 1 U/kg of A2 NTX significantly reversed the pain from 4 or 3 weeks, respectively (Fig. 3: C and D). Although the data were not shown, A2 NTX at 10 U/kg was observed to exhibit similar effects. It is interesting to notice that A2 NTX has analgesic effects in both peripheral and central neuropathic pain. Although the details of the anti-hyperalgesic effect are also unknown, it may share similar mechanisms with the inhibition of diabetic neuropathic pain. Further research should be done in the future.

In conclusion, in the present study, we found that A2 NTX, a pure component of botulinum toxin sub-

![Fig. 3.](image-url) Anti-neuropathic pain effects of A2 NTX in central spinal cord injury–induced neuropathic pain. A, B: A2 NTX (1 U/kg) was immediately applied into the injured spinal cord after spinal cord injury surgery. The rotarod tests, at 6 and 12 rpm, were performed during pre- to 14 days post–spinal cord injury. C, D: Thermal paw withdrawal tests and mechanical paw pressure tests were performed 2 to 8 weeks after spinal cord injury. SCI, spinal cord injury; PWL, paw withdrawal latency; PWT, paw withdrawal threshold. All data represent means ± S.E.M. from three to seven mice. *P < 0.05, vs. the spinal cord injury group.
type A2, exhibited a long-lasting anti-nociceptive effect in diabetes- and spinal cord injury–induced neuropathic pain models. The findings suggest that A2 NTX may become a potent candidate for treatment of neuropathic pain.

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References